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# Human Neuroblastoma Adaptation to Cobalt Chloride-Induced Hypoxia

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# **Human Neuroblastoma Adaptation to Cobalt Chloride-Induced Hypoxia**

Conor McAuliffe

Submitted in partial fulfillment of the requirements for the  
Degree of Master of Science in Biology from the  
Department of Biological Sciences of Seton Hall University  
May 2017

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## **Abstract**

Hypoxia is a frequent characteristic of the solid tumor microenvironment, which occurs when cancer cells lack adequate access to oxygen. By selecting for cells that can adapt to and grow in low oxygen conditions, tumor hypoxia contributes to a more aggressive and invasive cancer phenotype that portends a poor clinical outcome. While many aspects of the cellular response to hypoxia have been explored, the roles of some factors have not been fully explained. Cell signaling factors, including signal transducer and activator of transcription 3 (STAT3), the mu opioid receptor (MOR), and the delta opioid receptor (DOR), as well as changes in the mitochondrial membrane potential may play a role in cancer cell adaptation to hypoxia. Importantly, the hypoxia-related actions of STAT3 and the human opioid receptors have not been thoroughly researched within the context of human neuronal cancer cells. To investigate the roles of these proteins in hypoxic adaptation, we developed and tested a model of chemically induced hypoxia using neuronal cells. We administered the hypoxia mimetic, cobalt chloride, to a human neuroblastoma cell line. The preliminary data of flow cytometry suggested that cobalt-induced hypoxia increased the total amount of STAT3 in neuroblastoma cells, but decreased the amount of phosphorylated STAT3 in a time dependent pattern. The preliminary results using JC-1 staining with confocal microscopy indicated that cobalt exposure did not change mitochondrial membrane potential in surviving cells. RT-PCR (reverse transcription) analysis showed a decrease in MOR expression and no change in DOR expression. This decrease in expression lends support to an inhibitory role of MOR in neuronal adaptation to hypoxia. These results also implicated that survival of neuronal cells under cobalt treatment likely are independent of STAT3 tyrosine phosphorylation. Taken together this study provides a baseline for future use of this model to investigate hypoxia.



## Introduction

### *Tumor hypoxia*

Hypoxia is a common feature of the solid tumor microenvironment, in which cancer cells and their local tissue do not have adequate access to oxygen. The causes and effects of tumor hypoxia are multifaceted. Tumor hypoxia can be the result of at least three factors: irregular tumor vasculature, increased proliferation of cancer cells, and/ or anemia (Vaupel & Harrison, 2004). Due to a deregulation of endothelial regulatory signals, tumor-adjacent vascular tissue is often chaotically arranged and abnormally permeable (Baluk et al, 2005). These irregularities preclude normal perfusion of oxygen into the tissue, and hypoxia occurs.

Tumor cells exist in a hyper-proliferative state. This state increases their demand for both oxygen and nutrients, and when that demand outpaces the delivery capacity of existing blood vessels, hypoxia (low oxygenation) results. In addition, the rapid pace of cancer cell growth allows some tumor cells to grow beyond the distance that oxygen in tissue can diffuse to ( $<100\mu\text{m}$ ). Furthermore, chemotherapies and/or cancer cell signaling can reduce the capacity of blood to transport oxygen (Ludwig, et al, 1995). Thus treatment- and tumor-induced anemias cause the surrounding tissue to develop hypoxia. Within the tumor microenvironment, the balance between normoxia (normal oxygenation) and hypoxia temporally shifts as a function of the integration of hypoxia-inducing factors and hypoxia-response factors. As a result of this, tumors develop a spatially heterogeneous map of hypoxic regions (Jiang et al, 2012). The core of solid tumors tends to be anoxic and necrotic due to a complete lack of oxygen access. Cells more towards the tumor periphery of the tumor have greater access to oxygen, and exhibit lower levels of hypoxia (Evans et al, 2001; Evans et al, 2007).

From a clinical perspective, hypoxic conditions within the tumor microenvironment have been associated with resistance to cancer therapy, increased aggressiveness, invasiveness, and metastasis (Ruan et al, 2009). And it has been well documented that killing hypoxic tumor cells during cancer treatment requires an increased dosage of radiation as compared to normoxic tumor cells (Coleman, 1988). Any or all of these associations add evidence to the observed correlation between hypoxic tumors and poor clinical prognosis (Vaupel, 2008).

From a cellular perspective, hypoxia exerts selective pressure on a tumor cell population. The fate of any tumor cell encountering this hypoxic pressure is determined not only by the severity of hypoxia, but also by the cellular adaptability, which dictate whether the cell survives or induces apoptosis. The majority of tumor cells that are subjected to hypoxia die, but some cells are able to resist apoptosis and continue to proliferate. These surviving cells escape the constraint of hypoxia, gain a competitive advantage over hypoxia-sensitive cells, and are able to clonally expand; establishing colonies of tumor cells that persist under hypoxic conditions (Graeber et al, 1996).

Thus, hypoxia initiates an adverse cycle of tumor progression in which the microenvironment selects for cells that are capable of surviving hypoxia and may further promote proliferation and metastasis. Given the negative clinical implications of tumor hypoxia, understanding how cancer cells adapt to it is paramount in understanding the pathogenesis of cancer.

#### *The cellular response to hypoxia*

The cellular sensation of and response to hypoxia is mainly mediated through hypoxia inducible factor (HIF), which is upregulated in response to low oxygen, and thus in many cancers. HIF promotes the transcription of more than 100 downstream genes whose functions

include cell survival, proliferation, altered metabolism, and angiogenesis; processes that must be initiated in order for a cell to survive hypoxic conditions (Masoud & Li, 2015). Cancer cells in low oxygen regions of tumors can coopt this global hypoxia response system to ensure their survival and proliferation.

Independently or in cooperation with HIF, signal transducer and activator of transcription 3 (STAT3) may also play a role in the tumor cell adaptation to hypoxia. STAT3 is a latent cytoplasmic transcription factor that translocates to the nucleus to regulate gene expression, including the expression of many oncogenic proteins (Yu et al, 2009). The role of STAT3 in regulating the transcription of key oncogenes and its connection to various serine and tyrosine kinases suggest that STAT3 is a shared signaling node between several pathways, which all contribute to tumorigenesis (Yu & Jove, 2004). It is, therefore, unsurprising that STAT3 activity is increased in a broad spectrum of human cancers and cancer cell lines (Buettner et al, 2002). Additionally, in lung, breast, ovarian, pancreatic, and prostate cancer cell lines, hypoxia induced STAT3 activation (Noman et al, 2009; Pawlus et al, 2014; Selvendiran et al, 2009; Gray et al, 2005). These lines of evidence suggest that STAT3 allows for cellular adaptation to hypoxia by promoting the expression of proteins needed for survival in a low oxygen microenvironment.

#### *Hypoxia and human opioid receptors*

In addition to the role that STAT3 plays in promoting cellular adaptation to hypoxic conditions, the human mu and delta opioid receptors (hMOR and hDOR) have also been implicated in cancer progression (Singleton et al, 2015; Debruyne et al, 2010). Opioid receptor agonists, such as morphine, are widely used analgesics that are frequently a part of cancer-related pain management (Caraceni et al, 2012). Therefore, understanding the contribution that these receptors make to cancer progression is very clinically relevant. However, this research effort

has yielded somewhat contradictory results, possibly due to dosage differences, differences in route of administration, or differences between model system being used (Gach et al, 2011). For example, in cultured human neuroblastoma cells, hMOR activity promoted cancer cell survival by inhibiting apoptosis (Iglesias et al, 2003), while in cultured human glioma cells, inhibition of hDOR lead to an increase in apoptosis and a decrease in cell proliferation (Zhou et al, 2013).

Conversely, several anti-tumor effects have also been attributed to hMOR activity. In Lewis lung carcinoma cells xenografted into nude mice, hMOR activity suppressed angiogenesis, leading to an increase in tumor cell apoptosis (Koodie et al, 2010). Hatsukari et al also showed that morphine induced apoptosis in leukemia and adenocarcinoma cell lines, while contributing to increased necrosis in a breast cancer cell line (2007). These effects were diminished following naloxone (an opioid receptor antagonist) administration, suggesting that one or more opioid receptor was responsible for the increased apoptotic activity. Therefore, establishing a model of chemically inducible hypoxia within a neuronal cancer cell line that naturally expresses stable opioid receptor levels has the potential to reveal how these receptors participate in tumor cell adaptation to hypoxia.

#### *Chemically induced models of hypoxia*

Cellular hypoxia can be modeled using hypoxia mimetic compounds such as desferoxamine (DFO), cobalt chloride ( $\text{CoCl}_2$ ), and nickel chloride ( $\text{NiCl}_2$ ). Through somewhat different mechanisms, these compounds stabilize intracellular HIF by interfering with its proteasomal degradation (Ren et al, 2000; Yuan et al, 2003; Davidson et al, 2006). This allows HIF to accumulate within the cell and promote a hypoxic cellular phenotype. These hypoxia mimetic systems are experimentally advantageous as compared to culturing cells under “true”

hypoxic conditions (e.g. hypoxia chamber) because they are quicker to employ and are not susceptible to reoxygenation during the course of the experiment (Wu & Yotnda, 2011).

Cobalt has been used in numerous studies as a way of simulating a hypoxic cellular environment (Lee et al, 2001; Naves et al, 2013). Additionally, cobalt treatment induces reactive oxygen species (ROS) generation and has been shown to induce cell death in neuroblastoma and other cancer cell lines (Leonard et al, 2004; Stenger et al, 2011; Ardyanto et al, 2006). These effects of cobalt are well established, however its effects in cells that survive treatment and the cellular responses involved in enabling their survival are less clearly defined. And, while tumor hypoxia has been the focus of thorough study, and some of the key cellular response elements have been determined, many of the mechanisms underlying hypoxia-induced neuronal cellular changes have yet to be elucidated (Span & Bussink, 2015). In order to answer some of these questions, we used cobalt to model hypoxia in a human neuroblastoma cell line in order to study the adaptive responses that occurred in cells that survived this chemically induced hypoxia.

## Materials and Methods

### *Cell culture*

Human neuroblastoma (NMB) cells were grown in 75cm<sup>2</sup> tissue culture flasks (VWR, Radnor, PA), and provided with RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) in a 5% CO<sub>2</sub>-95% air, humidified incubator at 37°C. Cells were maintained with 0.05mg of Gentamicin Reagent/mL of medium (Life Technologies, Grand Island, NY) and 100 units of Penicillin Streptomycin/mL (Life Technologies) of medium.

### *Cobalt chloride treatment*

Cells were treated with 300μM cobalt chloride for 24 or 48hrs. For dose response assays, cobalt chloride was administered to cells at final concentrations of 100, 300, and 500μM for 24hrs.

### *Flow cytometry*

Control and cobalt-treated cells were washed to remove dead cells with serum-free RPMI, gently harvested with 0.25% trypsin-EDTA (1X) (Life Technologies), centrifuged, and resuspended in cold DPBS. NMB cells were fixed using BD Cytotfix-Fixation Buffer, and then resuspended in FACS buffer (2% FBS, 20% Versene in DPBS). Cell membranes were permeabilized with BD Phosflow-Perm Buffer III. Permeabilized cells were stained in the dark at 4°C using BD flow antibodies against pY705-STAT3 (Lot#4330520) tagged with Alexa Fluor 647 and total STAT3 (Lot#4106912) tagged with allophycocyanin (APC). Following incubation with stain, cells were centrifuged, excess stain was aspirated, and cells were resuspended in

FACS buffer. Fluorescent emission measurements were made in triplicate using a MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany) with lasers set to excite APC and Alexa Fluor 647 fluorophores. Analysis of flow cytometry data was performed using FlowJo (Ashland, OR).

#### *Trypan Blue Exclusion Assay*

Aliquots of live NMB cell suspensions that were harvested for flow cytometry protocol were used to determine relative cell viability. Briefly, control and cobalt-treated cells were stained (1:1 ratio) with Trypan Blue (Sigma-Aldrich) and the number of cells in each condition that excluded the dye was counted. Cell viability was expressed as percent of control.

#### *RNA extraction and RT-PCR*

Total RNA was extracted from NMB cells using TriReagent (Molecular Research Center, Cincinnati, OH). Extracted RNA was precipitated using chloroform and washed with cold 75% ethanol. RNA concentrations were measured at 260nm using a Beckman-Coulter UV/Vis Spectrophotometer. First strand cDNA synthesis was performed using random hexamer primers (Promega, Madison, WI) and a GeneAmp PCR System (Applied Biosystems), using the parameters: 37°C for 50 min, 70°C for 15 min. cDNA was stored at -80°C .

#### *PCR and DNA gel electrophoresis*

Equivalent quantities of total cDNA were added to each PCR reaction mixture. PCR amplifications of hMOR and hDOR were achieved by using human-specific primers: hMOR 5'-CTG GAA GGG CAG GGT ACT GGT GG-3' and 5'-CTG CCC CCA CGA ACG CCA GCA AT-3'; hDOR 5'- GTT CAC CAG CAT CTT CAC GCT C and 5'-CGG TCC TTC TCC TTG

GAG CCC-3'. Parameters for PCR amplification of hMOR and hDOR were 95°C for 1 min, 68°C for 30 sec (32 cycles for MOR; 25 cycles for DOR), 72°C for 30 sec. Human-specific  $\beta$ -actin primers (5'-CCT TCC TGG GCA TGG AGT CCT G-3' and 5'-TAC AGC GAG GCC AGG ATG G-3') were added as a loading control for 18 cycles. PCR products were run on 2% agarose/TAE gels containing ethidium bromide. Following electrophoresis, DNA gels images were taken under UV light with AlphaImager. The fluorescent intensities of target and h $\beta$ -actin bands were quantified using ImageQuant 5.2. Relative expression levels were normalized to mean h $\beta$ -actin signal and expressed as percent of control.

#### *Confocal microscopy*

NMB cells grown on coverslips were stained for mitochondrial membrane potential ( $\Delta\Psi_M$ ) by incubating JC-1 dye (Cayman Chemical, Ann Arbor, MI) (1:100) at 37°C in the dark for 30min. After incubation, the coverslips were washed twice with RPMI. Cells were imaged at 60X using a laser scanning confocal microscope (Fluoview 1000, Olympus, Tokyo, Japan) with 488 and 543nm lasers to detect FITC and Rhodamine Red X signals. As a positive control, cells were treated with 10 $\mu$ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a protonophore.

#### *Confocal image analysis*

A Rhodamine Red X filter was used to detect concentrated regions of J-aggregates and hyperpolarized  $\Delta\Psi_M$  on the red channel. Simultaneously, a FITC filter was used to detect regions of diffuse, non-aggregated JC-1 staining and depolarized  $\Delta\Psi_M$  on the green channel. Fluorescent intensities from both channels were measured using the line ROI tool in FV10-ASW software (Olympus). The ratio of average red channel (CHS2) signal/ average green channel (CHS1) signal for each ROI was calculated and compared.



### *Statistical analysis*

Statistical analysis was carried out using GraphPad Prism 5 with unpaired Student's t-test. Results were considered significant if  $p < 0.05$ .

## Results

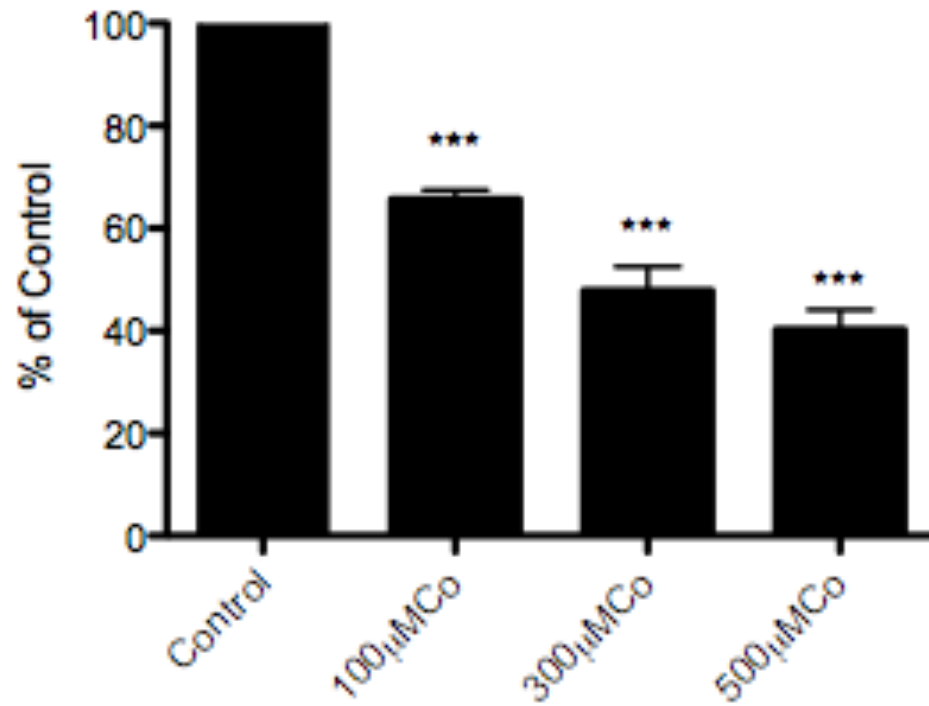
This study sought to establish a model of chemically induced hypoxia within a neuronal cell line. Specifically, cobalt chloride was used to generate a hypoxic phenotype within a neuroblastoma (NMB) cell line. Following 24 or 48hr cobalt treatment the surviving NMB cells and their hypoxia-related changes were analyzed. The results provide a foundation for use of this hypoxia model system in additional experiments aimed at unraveling the effect of hypoxia in neuronal cancers.

### *Cobalt treatment decreased NMB cell viability*

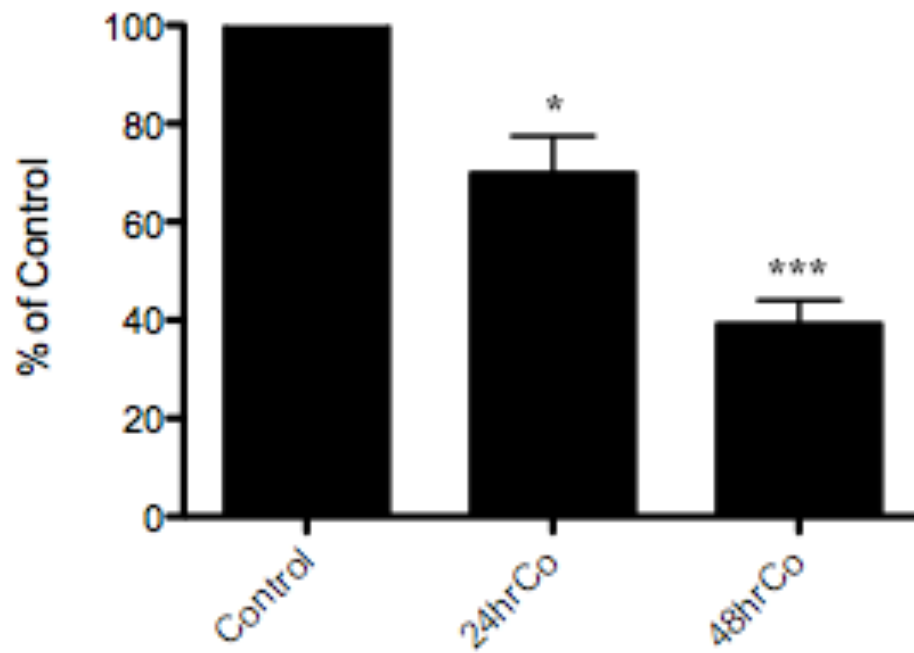
In order to establish a model system of cobalt-induced hypoxia within the NMB cell line, we first determined how cobalt administration affected overall cell viability. We used Trypan Blue to stain cell populations treated with increasing concentrations of cobalt for 24 or 48hrs to assess the viability of cells. Unviable cells retained Trypan Blue, while viable, living cells resisted the dye. By counting the number of unstained cells with a hemocytometer we determined the cell viability in each treatment condition. These measurements were then converted into percent of control (untreated cells).

Following 24hr treatment of NMB cells with an increase of cobalt concentrations, a significant dose-dependent effect on cell viability (Fig. 1) was observed. Treatment with 100 $\mu$ M cobalt decreased cell viability by ~34% (mean ~66% viability), 300 $\mu$ M decreased cell viability by ~52% (mean ~48% viability), and 500 $\mu$ M decreased cell viability by ~60% (mean ~40% viability). Since, 300 $\mu$ M cobalt treatment was sufficient to induce 50% cell death, we used this concentration to induce hypoxia in all other experiments.

NMB cells treated with 300 $\mu$ M cobalt also resulted in a significant decrease of cell viability in a time-dependent manner (Fig. 2). Twenty-four hour treatment resulted in ~30% decrease (~69% mean viability), while 48hr treatment decreased the mean cell viability to ~39% (~60% decrease) as compared to control. Regardless of treatment duration or concentration, there were still surviving neurons (Fig. 3), which were then used to study the adaptive responses.



**Figure 1:** NMB cells treated with cobalt for 24hrs significantly decreased cell viability in a dose-dependent manner as compared to control. Values represent the mean cell counts  $\pm$  SEM and are expressed as percent of control. Significance was determined using unpaired Student's t-test; \*\*\* $p < 0.0001$ .

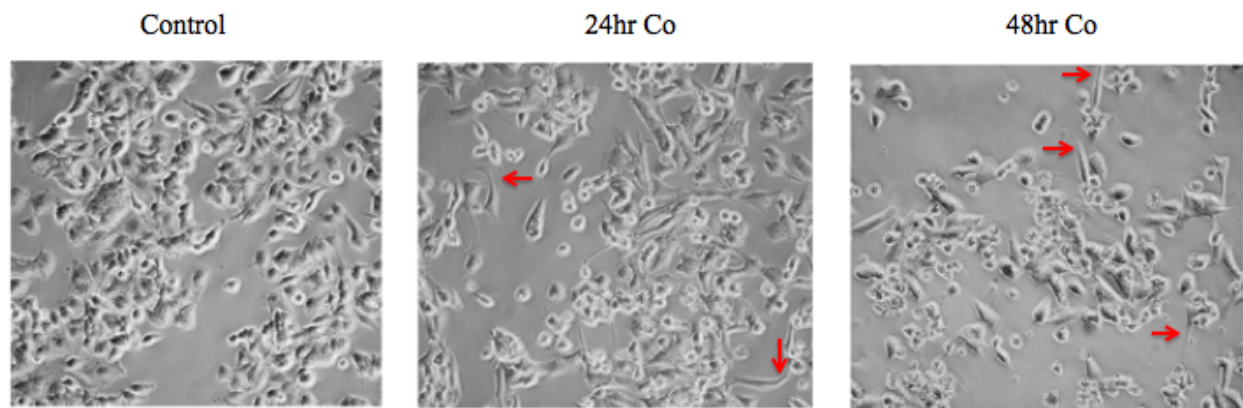


**Figure 2:** Cobalt treatment caused a time-dependent significant decrease in NB cell viability as compared to control. Values represent mean (+/- SEM) hemocytometer counts and are expressed as percent of control. Significance determined by unpaired Student's t-test; \* $p < 0.05$ , \*\*\* $p < 0.001$ .

*Cobalt treatment changed NMB cell morphology*

Hypoxia has been associated with morphological changes in murine neocortical neurons and human mesenchymal stem cells (Park et al, 1996; Zeng et al, 2011). Therefore, we examined NMB morphology under cobalt -induced hypoxia.

Along with the visual increase in the proportion of dead cells present in the treatment conditions, there was also a clear visual change in the morphology of the treated cells. Cells became more pronounced with 48hr cobalt treatment (Fig. 3), as compared to control cells, which were smaller and more rounded. A proportion of treated cells displayed elongated neuronal processes.



**Figure 3:** Treatment of NB cells with cobalt increased the length of neuronal processes (marked with red arrows), and the proportion of cells that displayed these processes. Also in these images is the increased number of dead cells in the 24 and 48hr treatment conditions.

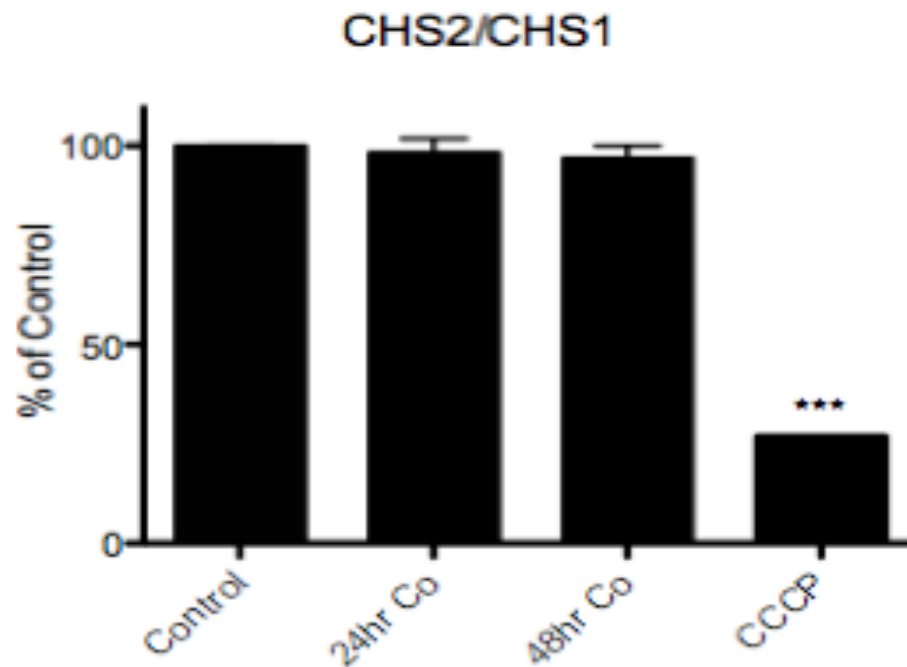
*Cobalt treatment did not affect  $\Delta\Psi_M$  of surviving NMB cells*

Previous research has implicated that cobalt treatment can result in mitochondrial membrane potential ( $\Delta\Psi_M$ ) collapse in human glioblastomas (Zeno et al, 2009). To determine if cobalt treatment can cause the depolarization of mitochondrial membrane potential, we therefore used laser scanning confocal microscopy to gauge differences in  $\Delta\Psi_M$  in NMB mitochondria labeled with a dichromatic ratiometric dye, JC-1. JC-1 dye has been used for this application in numerous studies and cell lines (Diaz et al, 1999; Perry et al, 2011). Briefly, JC-1 dye can exist within living cells in two forms, green monomeric form and red aggregate form. The red JC-1 aggregates accumulate in hyperpolarized (negatively charged) mitochondria, while green monomeric JC-1 highlights depolarized (more positively charged) mitochondria. In order to make qualitative and semi-quantitative measurements of  $\Delta\Psi_M$ , JC-1-dyed control and treated cells were stimulated using both 488 and 543 nm lasers and signals were detected with FITC (channel 1, CHS1) and Rhodamine Red X (channel 2, CHS2) filters. The ratio CHS2/CHS1 of each acquired image was calculated as a semi-quantitative measurement of  $\Delta\Psi_M$ .

We found that 24 or 48hr cobalt treatment does not significantly affect CHS2/CHS1 ratio as compared to that of control cells (Fig. 4). The mean CHS2/CHS1 value (expressed as percent of control) for 24hr cobalt treatment was ~98% and for 48hr treatment the mean value was ~97%. In order to demonstrate that JC-1 staining was sensitive to changes in  $\Delta\Psi_M$ , NMB mitochondria were purposefully depolarized using the protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP). In control cells incubated with JC-1 dye and CCCP,  $\Delta\Psi_M$  was significantly depolarized, leading to a quantitative decrease in the CHS2/CHS1 ratio. Visually, CCCP treatment resulted in a lack of accumulation of red JC-1 aggregates in depolarized



mitochondria with only diffuse green staining. Additionally, the elongated neuronal processes of treated NMB cells that were highlighted in Fig. 3 were also evident under confocal microscopy.



**Figure 4:** Mitochondrial membrane potential ( $\Delta\Psi_M$ ), as assessed by JC-1 ratiometric dye, is not affected by cobalt treatment. Mean values ( $\pm$  SEM) of the CHS2 (red channel)/CHS1 (green channel) ratios for control, 24hr Co, and 48hr Co, and CCCP. Significance was determined using unpaired Student's t-test; \*\*\* $p < 0.0001$ .

### *Analysis of STAT3 factor*

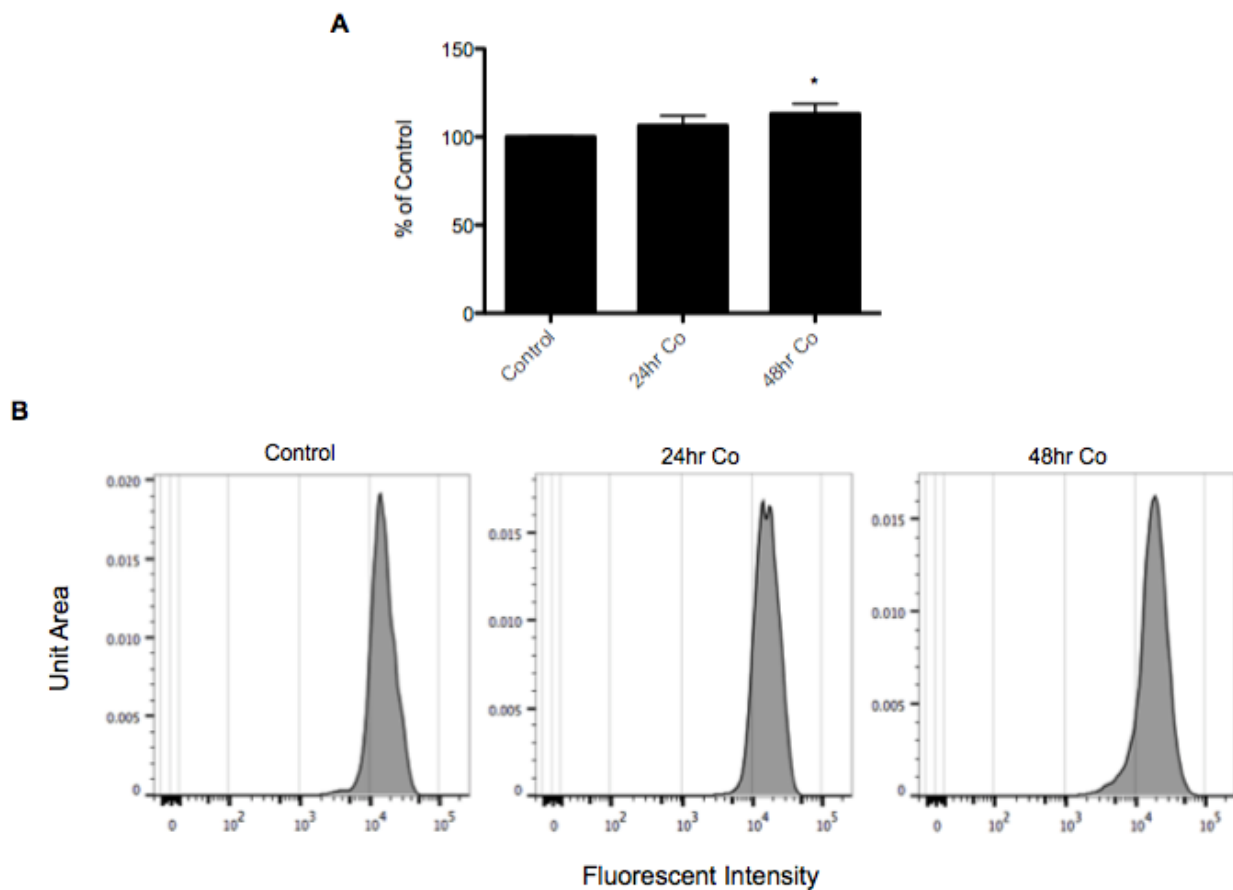
STAT3 is a transcription factor that requires phosphorylation of its tyrosine 705 (Y705) to translocate to the nucleus and regulate gene expression (Calo et al, 2003). Once activated, STAT3 promotes the expression of anti-apoptotic proteins such as Bcl-X<sub>L</sub>, survivin, and Mcl-1, cell cycle promoters including c-Myc and cyclin D1, and angiogenic proteins including VEGF (Ouédraogo et al, 2015). The role of STAT3 as a transcription factor for several proteins involved in the cellular response to hypoxia suggests that STAT3, especially phosphotyrosine STAT3 (pY705-STAT3), may be modulated by hypoxia. Furthermore, it has been demonstrated that phosphorylated STAT3 increases in response to cobalt stimulation in both adenocarcinoma and prostate carcinoma cell lines (Gray et al, 2005). In order to determine the role of STAT3 in cobalt treated neuronal cells, flow cytometry was used to analyze changes in total STAT3 levels and changes in the amount of phosphorylation at its Y705 residue in these surviving neurons.

### *Cobalt treatment increased total STAT3*

Control and cobalt treated cells were stained for total STAT3 protein using anti-STAT3 antibody tagged with allophycocyanin (APC) and analyzed using flow cytometry. All cell populations were gated on the parameters of forward scatter vs. side scatter (FSC vs. SSC) to exclude any remaining dead cells and/or cellular debris. The parameters of SSC vs. STAT3 fluorescence signal were then used to plot histograms.

Flow cytometry analysis of cobalt-treated cells revealed that these cells significantly increased their total level of STAT3 over 48hr treatment duration as compared to control cells (Fig. 5A). Twenty-four hour treatment resulted in a mean increase in total STAT3 of ~7% and 48hr treatment resulted in a significant mean increase in total STAT3 of ~13%. The increase in total STAT3 levels in treated cells is shown in the panel of histograms in Fig. 5B, as duration

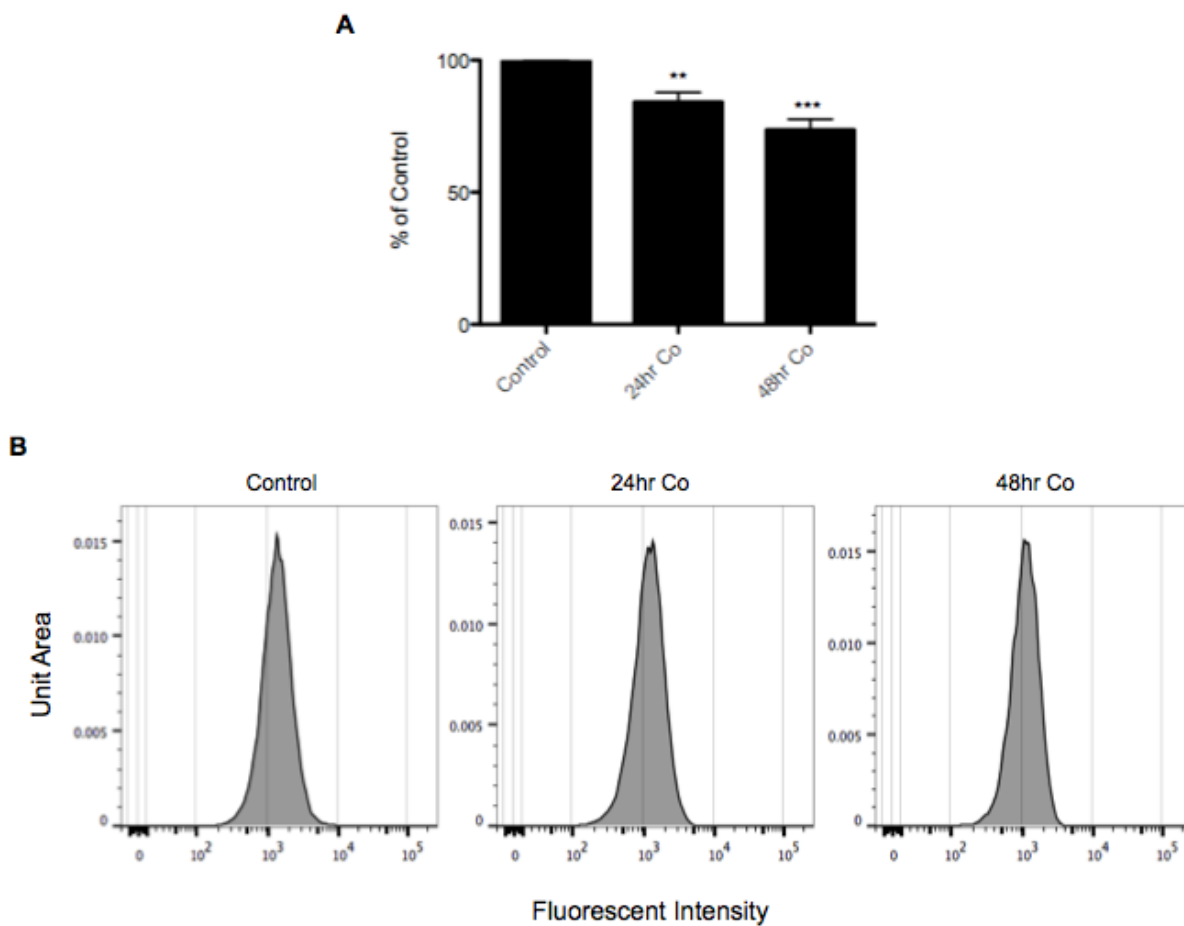
increases and the fluorescent signal becomes more intense. This shift in STAT3 fluorescence indicates that treated cells had an increased amount of total STAT3 as compared to that of control cells.



**Figure 5:** NMB cells significantly increased the amount of total STAT3 expressed over 48hr cobalt treatment. (A) Mean values (+/- SEM) of fluorescent intensities expressed as percent of control. Significance was determined using unpaired Student's t-test; \* $p < 0.05$ . (B) Histograms from a single set of representative measurements in which the mean fluorescent intensity increased as a result of cobalt treatment. Y-axis is normalized to area under the curve. X-axis is fluorescent intensity (log5 scale).

### *Cobalt treatment decreased pY705-STAT3*

Interestingly, the preliminary data showed that pY705-STAT3 levels were significantly decreased under various cobalt exposures (Fig. 6A). The same gating parameters (FSC vs. SSC) were used to include only intact cells. As compared to control, 24hr cobalt treatment significantly decreased the mean pY705-STAT3 signal to ~84% and 48hr treatment resulted in a decrease to a mean of ~74%. The histogram panel in Fig. 6B shows a time-dependent leftward shift in fluorescent intensity of pY705-STAT3. These preliminary data showed that total STAT3 level increased as the treatment duration increased, whereas STAT3 activation via Y705 decreased in response to cobalt challenge.

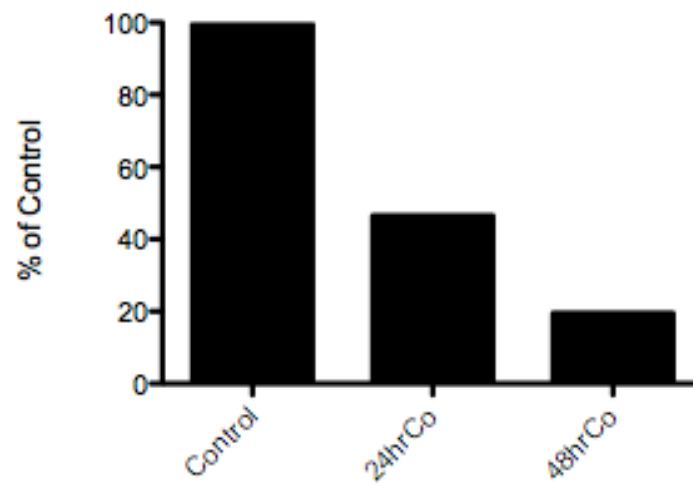


**Figure 6:** Cobalt treatment significantly decreased NMB pY705-STAT3 levels in a time dependent manner. (A) Mean values ( $\pm$  SEM) of fluorescent intensities expressed as percent of control. Significance was determined using unpaired Student's t-test; \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ . (B) Histograms from a single set of representative measurements in which the mean phosphotyrosine signal decreased as a result of cobalt treatment. Y-axis is normalized to area under the curve. X-axis is fluorescent intensity (log5 scale).

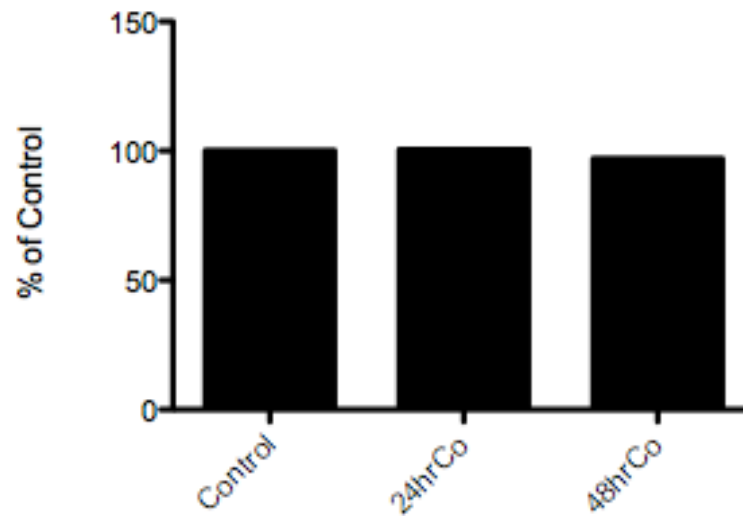
### *Effect of cobalt treatment on hMOR and hDOR expression*

NMB cells express all three endogenous opioid receptors (Baumhaker et al, 1994). As such, NMB cells are an ideal cell line for observing the cobalt-induced hypoxic effect on hMOR and hDOR expression. The preliminary data from RT-PCR analysis showed that NMB cells decreased expression of hMOR (Fig. 7) under the treatment. As compared to control, 24hr cobalt treatment resulted in a 54% decrease of hMOR expression, and 48hr treatment caused an 80% decrease in mean hMOR expression. There was no detectable change in hDOR expression as compared to that of control (Fig. 8).





**Figure 7:** hMOR expression decreased in NMB cells that survived cobalt treatment. The hMOR PCR product was analyzed using gel electrophoresis. The band intensities were normalized to mean h $\beta$ -actin signal. The data is expressed as the percent of control.



**Figure 8:** NMB hDOR expression did not change in response to cobalt treatment. The hDOR PCR product was analyzed using gel electrophoresis. The band intensities were normalized to h $\beta$ -actin signal. The data is expressed as the percent of control.

## Discussion

Hypoxia is a common feature of many solid tumors and can contribute to tumor progression, tumoral resistance to therapy, metastasis, and a poor clinical outcome. The causes of tumor hypoxia are well understood, but little research has been done on understanding the effects of hypoxia within a neuronal cancer cell line. Specifically, some of the factors that contribute to cell survival under hypoxic conditions are not fully understood. In order to address these questions, we established the basis for a model of chemically induced hypoxia using NMB cells with the hypoxia mimetic cobalt chloride. Utilizing this model, this study demonstrated that hMOR expression was decreased while an increase in total STAT3 accompanied by decreased phosphorylation at Y705 was detected.

By measuring significant decreases in overall NMB cell viability as a function of the duration and concentration of cobalt treatment, we established that this cell line is responsive to this hypoxia mimetic agent. These results agree with previous studies that show that cobalt chloride negatively affects cell viability. Cobalt has been reported to promote apoptosis in different cell lines, such as human acute myeloid leukemic cells, mouse embryonic fibroblasts, and human carcinoma cells (Huang et al, 2003; Vengellur & LaPres, 2004; Ardyanto et al, 2006). However, whether cobalt treatment can promote apoptosis in this NMB cell model system will need to be further investigated. Additionally, we detected distinct morphological changes that were characterized by elongation of neuronal processes in cobalt-treated cells. Other studies have also observed similar changes in cell morphology in response to cobalt hypoxia mimetic using mesenchymal stem cells (Zeng et al, 2011) and colon cancer cells (Lopez-Sanchez et al, 2014). Cobalt-induced morphological changes to neuronal cancer cells have not been previously reported.

In rat PC12 neuronal cells cobalt chloride induced apoptosis, which is accompanied by and likely initiated by a loss of mitochondrial membrane potential ( $\Delta\Psi_M$ ) (Jung & Kim, 2004; Jung et al, 2007). Using a similar method of measuring  $\Delta\Psi_M$ , we did not observe a change in  $\Delta\Psi_M$  of the surviving neurons. This discrepancy is actually unsurprising, as these surviving cells developed the adaptive responses under cobalt-induced hypoxia. To determine if  $\Delta\Psi_M$  disruption also causes apoptosis in NMB cells additional experiments will need to be performed that include apoptotic cells.

Given that some cobalt-treated cells experience a loss of  $\Delta\Psi_M$  before succumbing to apoptosis, it is possible that a resistance to cellular events that otherwise would induce pro-apoptotic  $\Delta\Psi_M$  changes, which we observed in surviving and attached cells, is part of the mechanism that allows some NMB cells to adapt to hypoxia. In fact, another group has recently demonstrated that apoptosis-resistant prostate and colon cancer cells preserved mitochondrial functionality as well as  $\Delta\Psi_M$  even after long-term hypoxia stimulation (Chiche et al, 2010). This report, as well as our results, suggest that a contributing factor of cancer cell adaptation to hypoxia may be the ability of some cell mitochondria to resist  $\Delta\Psi_M$  disruption, thereby forestalling apoptosis. More investigation will need to be performed to confirm this hypothesis, and to explore intracellular factors that may be responsible for mediating this mitochondrial activity.

An additional factor that may mediate NMB adaptation to cobalt-induced hypoxia is STAT3. In preliminary results, we observed a time-dependent increase in total STAT3 in response to cobalt administration, suggesting its involvement in the adaptive processes. Classically, STAT3 activation occurs via Y705 phosphorylation, which induces STAT3 homodimerization and directs it to the nucleus where it acts as a transcription factor for several

oncogenic cellular processes. Given that, membrane bound receptor tyrosine kinases, cytokine receptor-associated Janus Kinases (JAKs), and non-receptor tyrosine kinases have all been shown to phosphorylate Y705-STAT3 (Brantley & Benveniste, 2008), Y705-STAT3 is positioned as an intracellular node through which multiple signals are interpreted and passed. However, this study showed a decrease in the amount of phosphotyrosine STAT3 in a time-dependent manner in response to cobalt administration, suggesting that STAT3 activation via Y705 may not be involved in NMB adaptation to hypoxia. While these results need to be repeated and corroborated with different methods, they point to a role for STAT3 in response to cobalt-induced hypoxia that is not mediated via its critical tyrosine residue in this NMB cell model system.

Recently, several phosphotyrosine-independent roles for STAT3 have been proposed. These include maintaining  $\Delta\Psi_M$ , reducing ROS production, and mediating Ras-induced oncogenic transformation (Yan & Rincon, 2016). In order to promote these cellular processes mitochondrial localization of STAT3 and phosphorylation of its serine residue (S727) are required (Wegrzyn et al, 2009; Gough et al, 2009; Zhang et al, 2013). Disruption of  $\Delta\Psi_M$  and ROS generation are two well-documented effects of  $\text{CoCl}_2$  administration (Jung & Kim, 2004; Leonard et al, 2004). Therefore, S727 phosphorylation may also be involved in the development of adaptation. Taken together, this study along with various reports may implicate a bifurcated pathway of STAT3 activity: a classical pathway in which Y705 phosphorylation leads to STAT3 nuclear translocation and transcription regulation and a non-classical/mitochondrial pathway, which has been reported in both pancreatic cancer cells and glioma cells (Kang et al, 2012; Mandal et al, 2014).

Independent of the role of STAT3, this study also sought to clarify functions of hMOR and hDOR in response to cobalt-induced hypoxia. The endogenous expression of all three major opioid receptors in this NMB cell line made it ideal for measuring their expression changes under cobalt treatment. The preliminary RT-PCR data showed a time-dependent decrease in hMOR expression, while hDOR expression remained unchanged. These results point to a possible antagonistic function of hMOR in NMB survival, while also suggesting that hDOR may or may not be involved in the NMB response to hypoxia. Similar findings were also reported previously using the same NMB cell line with the hypoxia mimetic DFO (Cook et al, 2010; Candelora, 2014).

Overall, this study reported that the development of adaptive responses, including elongation of neuronal processes, increased levels of STAT3 and of hMOR expression, and decreased phosphorylation of Y705-STAT3, in surviving NMB cells under cobalt-induced hypoxia. Additional data sets will be added to further validate these preliminary results. Given that mechanistic differences between chemically induced hypoxia and “true” hypoxia exist (Huang et al, 2014), it will be useful to examine these adaptive response under actual low oxygen conditions in the future.

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